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XYLEM DYSFUNCTION IN *FICUS CARICA* INFECTED WITH WILT FUNGUS *CERATOCYSTIS FICICOLA* AND THE ROLE OF THE VECTOR BEETLE *EUWALLACEA INTERJECTUS*

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ABSTRACT

Ceratocystis ficicola causes serious wilt disease in many fig orchards in Japan. The transmission of this pathogen is thought to occur via soil to host roots, and an ambrosia beetle, *Euwallacea interjectus*, has been reported as a vector of the pathogen. Anatomical investigations were made on the disease development process with a particular focus on the responses of host tissue to the activities of the vector beetle and the pathogen. Living 26- and 8-year-old *Ficus carica* trees that were naturally infected with *C. ficicola* and had holes excavated by *E. interjectus* were used for analysis. Dark brown discoloration was observed in the sapwood of specimens with poor shoot elongation and slight leaf wilt at harvest. Discolored sapwood coincided with the distribution of hyphae of the pathogen, which was verified by the presence of conidiophores. Most of the beetle's gallery was distributed inside the discolored area. In the non-discolored sapwood adjacent to the border of the discolored area, some galleries were elongated and contained living new generation adults and larvae of *E. interjectus*. Hyphae of the pathogen and colored substances were identified also around those new galleries.

The present study showed that elongation of galleries by *E. interjectus* in the functional sapwood induces the wide distribution of the pathogen and contributes to the expansion of the discolored area in which vessels were dysfunctional. This process causes a shortage of water supply and wilting in the infected trees. *Euwallacea interjectus* must be contributing to the symptom development of this wilt disease.

Keywords: Wilt disease, sapwood, defense reaction, discoloration, ambrosia beetle.

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INTRODUCTION

The fungus *Ceratocystis* includes pathogens of many economically important tree diseases all over the world. In Japan, *Ceratocystis ficicola* Kajitani *et* Masuya, attacks cultivars of fig trees (*Ficus carica* L.) in orchards. This disease is a vascular wilt, and the pathogen was first reported as *Ceratocystis fimbriata* Ellis *et* Halsted in Japan (Kato *et al.* 1982). Kajitani and Masuya (2011) later identified it as a new species of the same genus. During the growing season of fig trees, which occurs from May to October in Japan, trees infected with this fungus start wilting from the top of the shoots, the leaves gradually turn yellow, defoliation occurs leaving just fruits, and the plants finally die (Kato *et al.* 1982; Shimizu *et al.* 1999; Nitta *et al.* 2005). Today, this disease occurs widely across fig plantation areas, most likely because of the distribution of infected saplings from diseased orchards (Kajitani *et al.* 1992; Shimizu *et al.* 1999; Togawa *et al.* 1999). Some farms abandoned their orchards because of the extensive damages caused by this wilt disease (Shimizu *et al.* 1999; Kajitani & Masuya 2011; Morita *et al.* 2012).

The dispersal process of *C. ficicola* has not been fully clarified. This pathogen is primarily recognized as a soil-borne plant disease because fig trees planted in soil polluted with the pathogen *C. ficicola* are easily infected (Kato *et al.* 1982). Therefore, application of fungicide to polluted soil and the breeding of resistant rootstocks have been tried during this decade (Hirota *et al.* 1984; Shimizu *et al.* 1999; Togawa *et al.* 1999; Yakushiji *et al.* 2012), but are not always effective to reduce the damage.

Kajitani (1996) found that an ambrosia beetle, *Euwallacea interjectus* (Blandford), contributed as a vector of this pathogen because it carries *C. ficicola* on its elytron (Kajitani 1999). This beetle species has a sib-mating system (inbreeding) and an extremely female-biased sex ratio: mating occurs after emergence in their galleries (nests in the host trees), and several male adults (brothers) of the new generation (offspring) copulate with many female adults (sisters) and then die without dispersal flight in search of new host trees because of their dwarf wings (H. Kajimura, personal observation). Female adults fly from dead trees in late March and mid-July (Kajitani 1999) and invade healthy tree trunks near the ground (Nitta *et al.* 2005). Morita *et al.* (2012) reported from field researches that this disease became epidemic in relation to the activity of vector beetles in Hiroshima Prefecture in western Japan. However, many researchers and orchard owners do not believe that the beetle is a vector of this disease, supposing instead that *E. interjectus* is a "secondary pest" and cannot affect healthy trees. Because the interaction between invasion by the beetle and development of the disease is still unclear, precautions against pathogen transmission by the insect are not taken in orchards.

The objective of this study is to clarify the host responses against the activities of *E. interjectus* and *C. ficicola* with anatomical techniques, and tracing the role of this insect in the process of disease development in fig trees.

MATERIALS AND METHODS

Specimens

Specimens of *Ficus carica* L. cv. Horaishi were obtained from two trees (tree A & tree B) in two adjacent orchards in Hiroshima Pref., Japan (latitude: 34° 16' 58",

longitude: 132° 45' 21"). Tree A (with a main trunk height of 43 cm, and a basal stem diameter of 29 cm) was 26 years old and harvested on 22 July, 2011. Many pinholes made by insects assumed to be *E. interjectus* were found on the lower trunk near the base on 10 August, 2010. The leaves of tree A had started wilting, but the tree was still alive. Tree B (main trunk height 39 cm, basal stem diameter 14 cm) was 8 years old and harvested on 17 July, 2012. Tree B had 6 pinholes on the trunk on 6 July, 2012, but did not show any wilt symptoms at harvest time.

Tree A was cut from the base and was dissected into discs of c. 7 cm thick (Fig. 1) just after the harvest. Tree B was cut in the same way as tree A. Discs from tree A and B were then brought back to the laboratory. The proportions of discoloration on the cross-cut areas were measured with software ImageJ (National Institutes of Health).

Microscopic observation

The surfaces of the discs obtained from the trunks and branches were observed under a binocular microscope (Nikon, SMZ1500). For light microscopy (Nikon, ECLIPSE 80i), xylem blocks (2×2×3 cm) that contained lesions or discoloration were cut from the discs. These xylem blocks were fixed in FAA (formalin, acetic acid, 50 % ethyl alcohol; 5:5:90, v/v) for one week and subsequently washed for one day under tap water.

With a sliding microtome, 20-μm-thick sections (transverse, tangential, and radial) were cut. Parts of the sections were mounted onto slides without staining to observe natural colors and cytological changes. Some sections were stained with safranin-fast green (Johansen 1940) for the observation of xylem cells, and others were stained with periodic acid-Schiff (PAS) and toluidine blue O (Feder & O'Brien 1968) for the observation of fungal hyphae.

The following aspects were given primary focus: a) the range of discolored xylem in the sapwood in relation to the invasion and gallery formation by the beetle, b) the distribution of fungal hyphae in the host tissue and the reaction of host cells, and c) the necrosis of parenchyma cells around the beetle galleries.

Fungal identification

Xylem blocks (length × radial depth × width = 5 cm × 5 cm × 5 mm) were cut from infected and discolored xylem of tree A and B with disposable knife blades and then washed with 70 % ethanol. Blocks were sterilized by short-period heating of the surface with a flame, kept moist in plastic bags with wet tissue paper, and incubated at room temperature (c. 25 °C). The perithecia formation of *Ceratocystis ficicola* was checked every day for two weeks.

RESULTS

External symptoms and macroscopic observation of infected fig trees

Pinholes found on the lower trunk of tree A were confirmed to be galleries made by *Euwallacea interjectus*. The fruits had not grown well since the spring of 2011, although this tree had not indicated any symptoms of wilt and defoliation during the previous year. Current-year shoots of tree A poorly elongated during spring, and were much shorter than those of unaffected trees. Leaves on the current-year shoots indi-

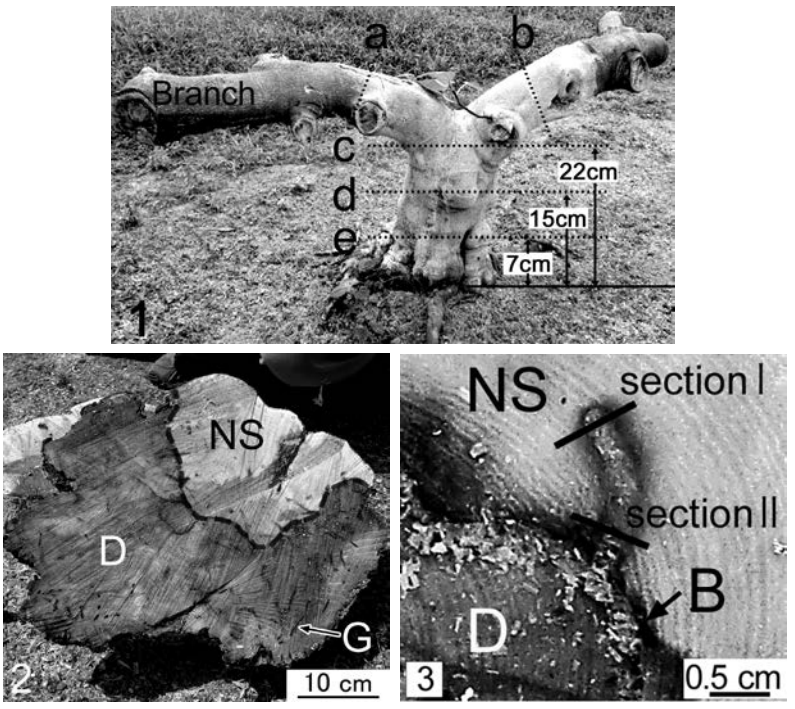

Figure 1. Trunk and basal branches of *Ficus carica*, with sampling positions indicated by the letters a–e. — Figure 2. Cross section of *F. carica* tree at 7 cm above ground naturally infected with *Ceratocystis ficicola*. (tree A, Fig. 1e). — Figure 3. A gallery of *Euwallacea interjectus* in the sapwood infected with *F. carica* (tree B, cross section). Section I and II: see Fig. 12. — NS = normal sapwood; D = discolored area; G = gallery of *E. interjectus*; B = border of discoloration.

cated slight wilting at harvest. These are characteristic symptoms of fig wilt caused by *Ceratocystis ficicola.*

Dark brown discoloration could be seen with the naked eye in a wide area of sapwood and a part of the phloem in the basal area of the trunk (Fig. 2). The proportion of discoloration on the crosscut area of the main stem was the greatest near the base of the trunk and reached 75 % at 7 cm, 59 % at 15 cm, and 44 % at 22 cm above the ground.

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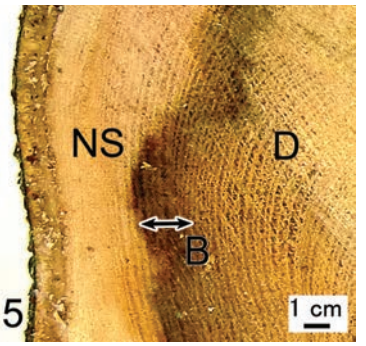
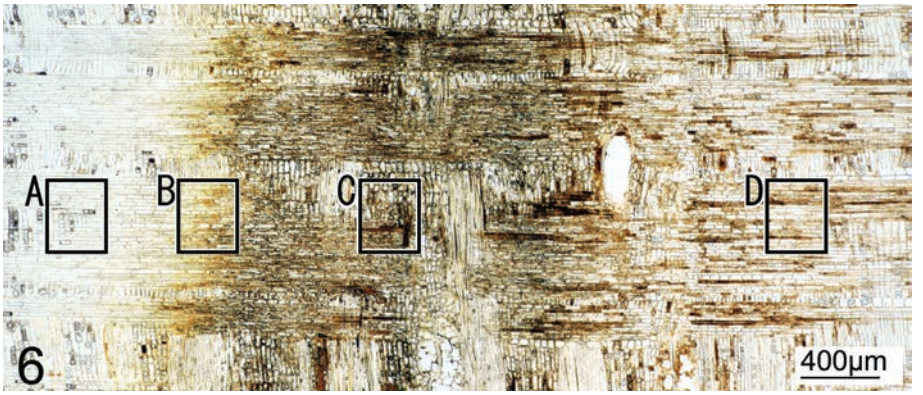
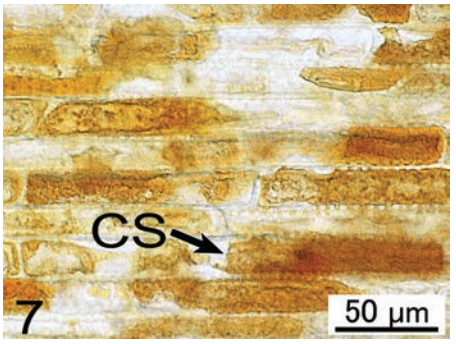
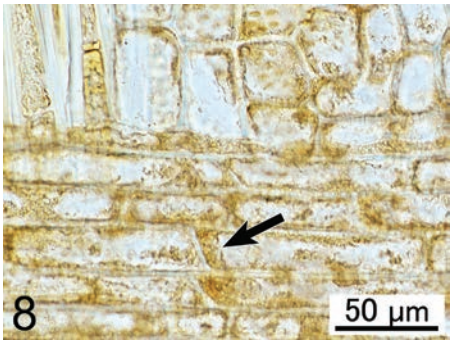
Figure 4–11. Macro- and microscopic views of *Ceratocystis ficicola* and *Ficus carica* sapwood. – 4: Perithecia of *C. ficicola* formed on the xylem block 5 days after sample collection. – 5: Transverse view of the darker band (arrow B) between discolored sapwood (D) and normal sapwood (NS). – 6: Radial section of the darker band B in Fig. 5. Enlarged images of areas B to D are cited in Fig. 7 to 9, respectively. – 7: Cells with colored substances (arrow CS) in the outermost part of the border, the area B of Fig. 6. – 8: Deeply pigmented non-nucleated parenchyma cells (arrow) in area C of Fig. 6. – 9: Hyphae (arrow H) observed in the discolored sapwood in area D of Fig. 6, conidiospoes (C), and colored substances (CS). – 10 & 11: Conidiospores of *C. ficicola* in the discolored sapwood in area D of Fig. 6.

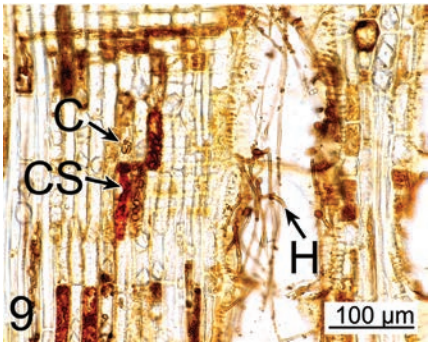

4
1 mm


NS
D
B
5
1 cm


A
B
C
D
6
400μm


CS
7
50 μm


8
50 μm


C
CS
H
9
100 μm


10
25 μm


11
25 μm

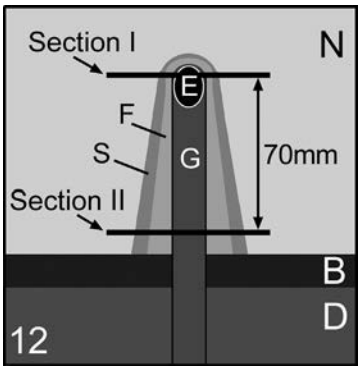


Figure 12. Schematic diagram of a newly-formed gallery of *Euwallacea interjectus* in the normal sapwood (tree A). Microscopic view of Section I and Section II are shown in Fig. 14 to 17. – N: normal sapwood; E: living adult females of *E. interjectus*; F: area of fungal distribution; S: area of slightly yellow-stained tissue; G: gallery of *E. interjectus*; B: border of discoloration; D: discolored area.

At the base of two boughs, the discoloration was 31% and 41%. In the disc obtained from the main stem from 15 cm to 22 cm above ground, 39 entrance holes excavated by *E. interjectus* were found. Discolored sapwood indicated in Figure 2 covered the area of most galleries made by the beetle. Some of the galleries were found to penetrate through the border of discolored and non-discolored sapwood (Fig. 3).

The trunk of tree B, which was obtained from a different orchard, adjacent to the harvest site of tree A, had 6 holes made by *E. interjectus*. The beetle must have invaded the tree during the period of early March to late July of 2012 because no beetle attack was found in early March. Internodes of the current year's branch were shorter in this specimen than they were in tree A. However, tree B did not show wilt symptoms at harvest time. The proportion of discoloration on the crosscut area was up to 52% in the main stem.

In tree A and B, cambial necrosis was observed adjacent to the discolored xylem area. Cambial necrosis was not observed in the branches showing slightly wilting leaves.

Distribution of Ceratocystis ficicola in the host tissue

On the xylem blocks from the discolored xylem of both trees, perithecia of *C. ficicola* formed about four to seven days after incubation at room temperature (Fig. 4). It confirmed that the harvested trees had been infected with *C. ficicola* in the orchards.

On the crosscut surface of trunk discs from both trees, dark bands measuring 5 to 20 mm wide were observed with the naked eye between the discolored sapwood and the normal, functional sapwood of whitish color (Fig. 5). Detailed microscopic observation focused on the black band and the areas on both sides toward the discolored

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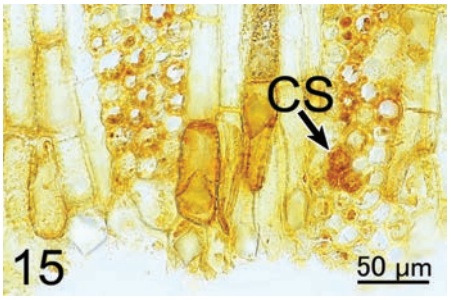
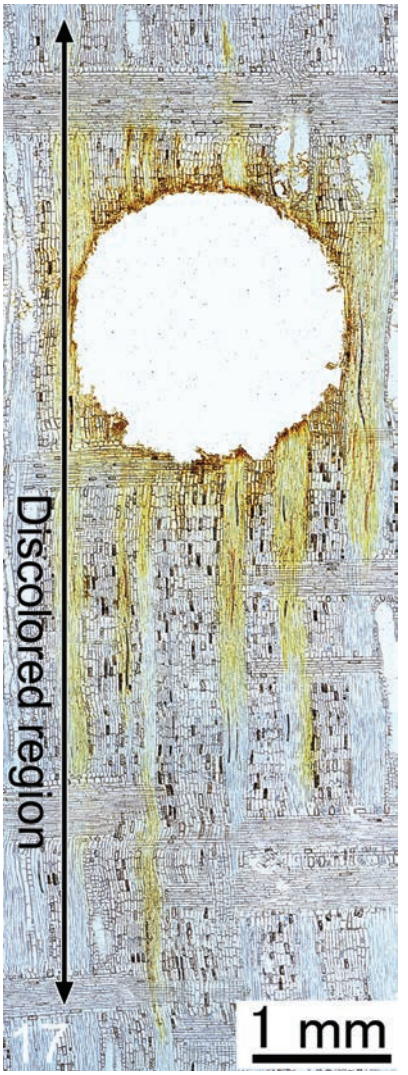
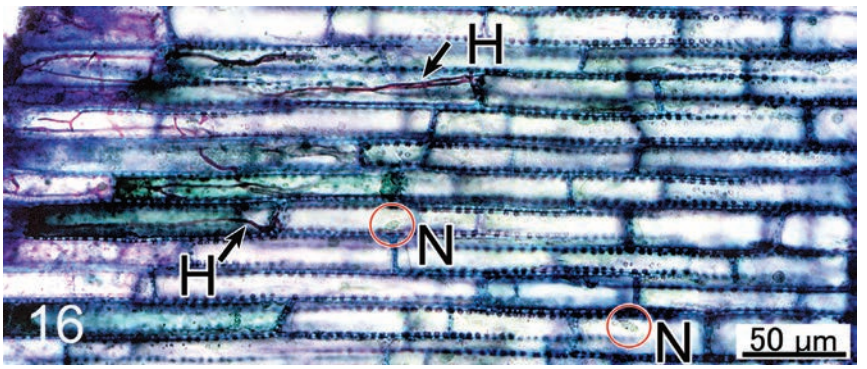
Figure 13–17. Host reaction associated with the activities of *Euwallacea interjectus*. – 13: A new generation of female adult *E. interjectus* with bright colour. – 14: Beetle's gallery in the functional sapwood, close to its end (Section I in Fig. 12). – 15: Accumulation of brownish-yellow substances in parenchyma cells (arrow CS) and staining of surrounding cell walls. – 16: Hyphae elongating into ray parenchyma cells (arrows H) and living cells with nucleus (circled N). The section was stained with periodic acid-Schiff (PAS) and toluidine blue O. – 17: Older parts of the same gallery with wider discoloration (= Section II in Fig. 12).


13
1 mm


Discolored region
14
1 mm


CS
15
50 µm


Discolored region
17
1 mm


H
N
H
16
N
50 µm

area and the normal whitish sapwood. As indicated in Figure 6, detailed microscopic observations were made along the radius from A to D, based on the condition of the discoloration. Area A is normal sapwood in which ray parenchyma cells contain big round nuclei similar to the host tissue without infection. Area B is the outermost area of discoloration (Fig. 6B) located just outside the black bands. The ray parenchyma cells were occluded by pale yellow droplets (Fig. 7, arrow) and some cells have nuclei in this area. Area C is the center of the black bands (Fig. 6C). Ray parenchyma cells in this area contained a small amount of dark brown substance (Fig. 8, arrow), had no nuclei, and were necrotic. Area D is inside the discolored xylem (Fig. 6D). All parenchyma cells in area D contained no nuclei and were necrotic. Colored substances had accumulated in some of the dead cells and fiber lumina (Fig. 9). In areas A and B, no fungal hyphae were observed. In areas C and D, hyphae were observed in vessels, fibers, and axial and ray parenchyma cells (Fig. 9, arrow). In area D, cylindrical or slightly lageniform conidia of *C. ficicola* occurred (Fig. 10 & 11). Tyloses were not observed in the vessels occupied by the fungal hyphae, although they were found in the vessels near the boundaries between functional sapwood and infected wood.

Xylem discoloration associated with gallery elongation and fungal distribution

One of the new galleries found in the pale-colored (sound) sapwood in tree A is shown diagrammatically in Figure 12. Living, newly-emerged adult females of *Euwallacea interjectus* (Fig. 13) were found at the end of new galleries under formation in the normal sapwood of tree A. In tree B, living females and larvae of *E. interjectus* were found in galleries that extended from the discolored area into normal sapwood (Fig. 3). Section I in Figure 12 indicates the end of the gallery where the females of *E. interjectus* occurred. Under the light microscope, a yellowish stain was observed in the vessel lumina, fiber walls, and axial and ray parenchyma cell walls surrounding the gallery wall (Fig. 14, arrow). Dark yellow-colored droplets were observed scattered in the cytoplasm of some parenchyma cells in this area (Fig. 15, arrow). Inside the stained area (Fig. 14), hyphae were observed in the lumina of vessels, fibers, and parenchyma cells that had been mechanically broken by the beetle. In the ray tissue, fungal hyphae are invading living parenchyma cells that contain big round nuclei similar to cells in the unaffected tissue (Fig. 16). In section II (Fig. 12), the cell walls were stained a darker brown color (Fig. 17. arrow), and the parenchyma cells contained darker brown droplets than the cells of section I. The ranges of stained areas and fungal distribution were larger in section II and formed earlier than in section I.

DISCUSSION

The mortality or survival of trees infected with wilt disease is determined by the degree of the blockage of xylem sap ascent in the trunk (Kuroda 2001, 2005; Kuroda *et al.* 2006). The xylem discoloration occurs in the hardwood xylem as a defense mechanism of trees for protection against microbial invasion (Shigo & Hillis 1973; Hillis 1987) and is called wound heartwood or pathological heartwood (Hillis 1987). It is well known that xylem dysfunction progresses as the discolored sapwood expands in the

diseased hosts (Kuroda 1996). All parenchyma cells are necrotic and all vessels are dysfunctional in wound heartwood as in the case of normal heartwood (Holbrook & Zwieniecki 2005). In the present investigation, the trunks of the fig trees infected with *Ceratocystis ficicola* had discolored up to 78% of the crosscut area at the base when wilt symptoms began. This suggests that water conduction had decreased severely and the wilt symptom started due to the deficit of water supply during the hot and dry summer season. This progression is similar as in other wilt diseases of *Quercus, Picea* and *Pinus* species (Kuroda 2001, 2005, 2008). Cambial necrosis, which was observed in a part of the trunk circumference and was not observed in branches around slightly wilting leaves, is not the direct cause of the wilt symptom as confirmed in other wilt diseases caused by *Ceratocystis* species (Kuroda 2005).

In the part of the xylem without gallery formation, successful compartmentalization, a boundary-setting to minimize further damage by the microorganisms (Shigo & Marx 1977; Shigo 1984) was observed. The boundary between discolored and sound sapwood, where the parenchyma cells occluded by pale yellow droplets correspond to the 'reaction zone' defined by Shain: parenchyma cells in the 'reaction zones' are filled with antibiotic compounds, such as polyphenols, that are effective in preventing the spread of pathogens in living trees (Shain 1967, 1971, 1979). Hyphae were not observed in the normal sapwood outside this zone. The present observations suggest that the boundary between discolored and sound sapwood is effective to prevent the spread of pathogens as hypothesized by Shain.

It was very significant to note that an ambrosia beetle, *Euwallacea interjectus* was found excavating galleries from discolored xylem into normal sapwood devoid of fungi. *Euwallacea interjectus* adults could enlarge their activity range from discolored, necrotic xylem into sound sapwood that contains living parenchyma cells. This type of gallery extension must have assisted wider distribution of *C. ficicola* in the trunk. When a microorganism invades a tree, parenchyma cells synthesize and accumulate antibiotic compounds consisting of phenolic substances such as terpenoids, stilbenoids, and alkaloids (Kemp & Burden 1986; Hillis 1987) and prevent the distribution of pathogens. However, this defense system could not block the extension of beetle galleries. Around the newly-extended galleries in the living sapwood, hyphae of the pathogen were found distributing in the lumina of broken cells from the gallery. The extension of a gallery sets up a new entrance for hyphae into sound tissue that has not yet experienced a defense reaction. *Ceratocystis ficicola* is thus successful in crossing through the defensive boundary zone by using the galleries of *E. interjectus*.

As a result of fungal invasion from necrotic and dysfunctional areas into living sapwood through the beetle's gallery, the areas of defense reaction expanded in the sapwood and induced enlargement of the "wound heartwood". This relationship between vector beetle *E. interjectus* and *C. ficicola* is similar to that of ambrosia beetle, *Platypus quercivorus* (Murayama), a vector of the Japanese oak wilt pathogen *Raffaelea quercivora* Kubono & Ito. Wilting of oak trees also occurs because of water deficit due to the expansion of discolored sapwood following the dense gallery formation by *P. quercivorus* (Kuroda 2001; Esaki *et al.* 2004; Kinuura & Kobayashi 2006). In the case of Japanese oak wilt, survival or mortality of oak trees infected with the pathogen

is determined during one summer (Kobayashi & Ueda 2005). In contrast, *E. interjectus,* once colonizing the trunk of a fig tree continues to reside in the same living tree for a few years as long as the condition in the trunk is suitable for their reproduction (H. Kajimura, personal observation). Therefore, in the fig trees, enlargement of discolored and dysfunctional sapwood gradually progresses in association with the continuous activity of *E. interjectus*. Morita *et al.* (2012) reported that fig trees did not die for two years after the first invasion of *E. interjectus* into the stems and the simultaneous infection of the pathogen. The delay of wilt symptoms in fig trees of more than one year from the first infestation of *E. interjectus* seems to have induced a negative opinion on the beetle's contribution to the disease development. The present study showed the role of the vector beetle in spreading pathogen in the healthy sapwood and its contribution to the enlargement of the dysfunctional area. Judging from the observation of Japanese oak wilt (Kuroda & Yamada 1996), wilt symptoms do not always occur as long as water is supplied to the shoots, even though sap flow has diminished compared to healthy trees. It is logical that infected trees take years from the initial attack by *E. interjectus* to reach mortality, judging from the slow and gradual enlargement of xylem discoloration until the dysfunctional area covers most of the sapwood in the basal area of trunks. Although many ambrosia beetles have been classified as secondary beetles that use dead, almost dead, or fallen trees for their reproduction (Furniss & Carolin 1977), *E. interjectus* female adults were evidently using living trees for their reproduction. Therefore, it does not seem correct that *E. interjectus* is classified as a typical secondary attacker. In recent years, some excavations in living trees by beetles were reported, like *Platypus quercivorus* (Kühnholz 2001). It suggests that some beetles associated with pathogenic microorganisms may utilize the trees that look healthy but are physiologically stressed. For an analysis of host conditions suitable for the reproduction of *E. interjectus*, detailed investigations on the invasion strategy of mother beetles into fig trees will be necessary.

The present investigation showed that xylem dysfunction caused by the wide distribution of *C. ficicola* in the sapwood is the cause of the wilt symptom of fig trees. Although this disease is sometimes called Ceratocystis canker, the pathogen does not form a canker but causes a wilt. In addition, the results support previous reports by Kajitani (1996) and Morita *et al.* (2012) that claimed that *E. interjectus* is a vector of *C. ficicola*. In order to clarify the responses of host cells to *C. ficicola*, we are currently conducting inoculation experiments on fig saplings.

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